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Recipient age and weight affect chronic allograft nephropathy in rats

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1 Introduction

The short-term results after clinical organ transplantation have improved progressively over the last decades. Thanks to refinements in tissue typing, advancements in organ preservation, operative techniques and auxiliary care, more effective immunosupressive agents, and better monitoring after engraftment. For example, one year survival of cadaveric kidneys has increased from approximately 50% by the end of the 1960s, to about 85% nowdays (Gjertson DW. 1996), and for living-related kidneys from 80% to 90-95% (Terasaki PI et al. 1993). Despite this impressive progress, however, it has become clear that clinical transplantation has not achieved its goal as a long-term treatment.

The two largest problems in renal transplantation are organ shortage and late graft loss with chronic allograft nephropathy (CAN) being the main causes (Paul LC. 1995).

1.1 Definition of chronic allograft nephropathy

Chronic allograft nephropathy (CAN) or chronic rejection is the most common cause of late renal allograft loss. It is defined as a state of impaired renal allograft function at least 3 months posttransplantation, independent of acute rejection, overt drug toxicity, and recurrent or de novo disease (Halloran PF et al. 1999). CAN is characterized by functional impairment with a nonspecific pathology: tubular atrophy, interstitial fibrosis, and fibrous intimal thickening in the arteries, with variable glomerular lesions. Both alloantigen-dependent and alloantigen-independent mechanisms may participate in the development of this progressive renal graft dysfunction, eventually leading to similar histologic features and clinical picture. CAN has been regarded as a synonym for chronic renal allograft rejection in many studies of late graft dysfunction. Although alloantigen independent mechanisms may play an important

role in the pathogenesis of progressive graft failure, the term chronic rejection is still used, both to outline the importance of immunologic mechanisms, even in events triggered by alloantigen-independent factors, and to avoid any confusion with the chronic nephropathy caused by the calcineurin inhibitors, cyclosporine and tacrolimus.

1.2 Histology of chronic allograft nephropathy

Chronic allograft nephropathy is characterized in the Banff classification by chronic transplant glomerulopathy, mesangial cell proliferation, mesangial matrix increase and peripheral mesangial interposition with thickening of glomerular capillary loops, interstitial fibrosis and tubular atrophy, as well as proliferation and thickening of the vascular intima. The CAN triad-tubular atrophy, interstitial fibrosis, and fibrous intimal thickening-is the basis for the Banff classification of CAN (Solez K et al. 1993).

Histologically, CAN is characterized by fibrous intimal thickening of arteriolar and arterial vessels, usually associated with glomerular and tubulointerstitial changes. The arterial intimal fibrosis (also called transplant arteriopathy) may affect small preglomerular arterioles as well as interlobular arteries and even main renal artery. Initially, there are proliferative changes of intimal and smooth muscle cells, followed by progressive vascular sclerosis and obliteration caused by extracellular matrix deposition (Colvin RB. 1996). The glomeruli may show an increase in mesangial cell and matrix, thickening and duplication of glomerular basement membrane, with scarring and adhesion (Habib R et al. 1993). These lesions are also called transplant glomerulopathy. There is a diffuse interstitial lymphocytic infiltration and various degrees of interstitial and tubular atrophy. The peritubular capillaries may show thickening and multilayering of the basement membrane, which appear to be specific (Monga G et al.

1992).

1.3 Clinical manifestations of chronic allograft nephropathy

Clinically, CAN is characterized by a progressive deterioration of kidney allograft function. Proteinuria and hypertension are often associated with CAN. The onset of proteinuria is generally considered as highly suspicious for an underlying CAN. Usually proteinuria ranges between 1 and 2 g per day (Hostetter TH. 1994), but it may be in a nephrotic range in the case of an underlying transplant glomerulopathy (First MR et al. 1984). A correlation between the level of proteinuria and CAN has been found. Arterial hypertension is extremely common in CAN. An association between the severity of hypertension and the degree of histological (Kasiske BL et al. 1991) damage has been reported.

1.4 Risk factors for chronic allograft nephropathy

Both alloantigen-dependent and alloantigen-independent mechanisms are involved. Two hypotheses have been suggested to explain the etiology of CAN: the first is that the phenomenon is primarily an alloantigen-dependent event influenced by early acute immunological injury to the graft and by later ongoing host alloresponsiveness; the second is that donor-associated alloantigen-independent factors and those surrounding the engraftment procedure predispose for chronic changes (Halloran PF et al. 1999). As these mechanisms are probably often interwined and it is difficult to separate the relative impact of alloantigen-dependent or alloantigen independent factors on the development and progression of CAN. The most commonly accepted risk factors are histoincompatibility, acute rejection, infections (particularly cytomegalovirus infection), drug toxicity, patient, donor age, hyperlipidemia,

hypertension, and initial ischemia reperfusion injury (Szabo A et al. 1998).

1.5 Mechanisms of chronic allograft nephropathy

CAN has been considered as a process of scar formation, resulting in the disruption of the normal tissue architecture and function. Repetitive tissue injury over a short period of time may result in excessive production of fibrogenic cytokines and excessive scar formation, with or without decreased breakdown of the deposited extracellular matrix material, as shown in other renal diseases with interstitial fibrosis (Eddy AA 1996). Hypotheses have been proposed to explain the mechanisms that are involved in late renal allograft loss.

(1) Maladaptive response to early injury-induced nephron loss: A growing body of data implicates initial injury processes, both alloantigen-dependent and alloantigen-independent, are associated with extensive nephron loss as a significant determinant of progressive graft injury of late renal allograft failure. The development of progressive glomerulosclerosis has been expected to reinforce the stimulus-inducing hyperfiltration and hypertrophy in less affected nephrons, thereby establishing a self-sustaining vicious cycle (Azuma H, et al, 1997). Increased glomerular capillary hydraulic pressure ($\uparrow P_{GC}$) (Kingma I et al. 1993, Junaid A et al. 1994, Mackenzie HS, et al. 1996) and glomerular hypertension (Junaid A, et al, 1994; Mackenzie HS, et al, 1996) have been observed in the F344→LEW rat model of kidney transplantation, suggesting that chronic glomerular injury in this model is driven, at least in part, by glomerular hemodynamic factors. Evidence supporting this view came from studies showing that restoring total recipient kidney mass essentially normalized P_{GC} and markedly reduced chronic allograft injury (Mackenzie HS et al. 1996). The renoprotective effects of losartan, a specific angiotensin II type I receptor antagonist, have been proven to be associated with lowered P_{GC}, inhibition of macrophage chemoattractants and recruitment, and

suppression of macrophage-associated cytokines at 20 weeks posttransplantation. This suggests that chronic renal allograft injury in F344→LEW rats is, to a large extent, mediated by angiotensin II-dependent mechanisms (Ziai F et al. 2000).

(2) Ongoing alloresponsiveness: Tissue damage may expose antigens that are normally not accessible or recognized by the immune system, and the proinflammatory milieu of a damaged and inflamed graft may, thus, enhance immune responses against a variety of such tissue antigens (Matzinger P 1994). Local injury and inflammation enhance the production of chemokines and the expression of chemokine receptors, and, thus, recruit antigen-presenting cells, increase MHC expression, and enhance costimulation to favor active immune responsiveness. Alloantibodies against graft glomerular and tubular basement membrane antigens as well as autoantibodies against mesangial focal adhesion plaque proteins (Paul LC et al. 1998) have been found in animals with chronic allograft nephropathy. These antibodies against basement membranes could cause functional impairment and/or initiate an antibody-mediated inflammatory response. Similarly, antibodies against mesangial cells may cause apoptosis or complement-dependent mesangiolysis, or they may activate C5b-9 to interact with the cell membrane, and mediate inflammation (Paul LC et al. 1999). Furthermore, it is conceivable that antibodies to focal adhesion plaques interfere with the cellular signal transduction cascade as focal adhesion plaques are sites for integrin-dependent control of a variety of second messengers modulated by the protein, tyrosine kinase $PP125^{FAK}$ (Haneda M et al. 1995). Tyrosine phosphorylation and activation of $PP125^{FAK}$ are associated with the activation of a variety of growth factors and the binding of antibodies to focal adhesion complexes could disrupt the normal balance between cell proliferation and matrix formation by acting either as an activating ligand or stabilizing the ligand-receptor interaction. The paradigm of an immune response against newly exposed, damaged, or activated graft cells and some of their secretary products could explain the clinical correlation of graft injury and CAN.

(3) Apoptosis: The physical or three-dimensional extracellular matrix structures that determine and maintain the organization of normal tissues may get damaged during various forms of organ damage, but it cannot be repaired by any of the cells involved in the tissue repair processes. Whereas a number of anatomic structures such as blood vessels can regenerate after extensive damage, other structures are dependent on an intact extracellular matrix framework. Tubular epithelial cells, for example, must find an intact tubular basement membrane upon which to attach their integrins, to proliferate and to organize their polarity to the tubular lumen, but if they do not find this structure they will undergo apoptosis (Frisch SM, et al, 1994). Thus, the disruption of the extracellular matrix framework may result in an inability to restore or maintain the graft parenchymal architecture.

(4) Cell senescence: The effects of both non-immune and immune interaction have been hypothesized to accelerate the process of senescence in cells to heal and to resolve injury (Halloran PF et al. 1999). Loss of the ability of key cell populations in transplanted organs—including vascular endothelial cells, and renal and bronchial epithelial cells—to proliferate and repair injury leads to focal atrophy, fibrosis, and diminished reserve and function. The aging kidney shows arterial intimal thickening, focal tubular atrophy, interstitial fibrosis, sclerosis of glomeruli and patchy infiltration of chronic inflammatory cells. All these changes are characteristics of CAN (Epstein M 1996). In renal allografts, inflammation caused by unresolved injury or rejection induces stress that may eventually exhaust repair capacity (Halloran PF et al. 1999). Inflammation and fibrosis are normally under control of the healing processes in a healthy tissue, but senescent or exhausted cells may fail to control fibrosis. Fibrosis arises, according to this hypothesis, from an exhaustion of graft parenchymal cells after a multitude of stresses.

(5) Macrophages, cytokines, and growth factors: Whether the etiological factor or trauma is alloantigen-independent or alloantigen-dependent in origin, all will ultimately result in graft

inflammation. Proinflammatory cytokines and profibrotic growth factors are thought to play a major role in the pathogenesis of chronic renal allograft injury (Diamond JR, et al, 1992; Hancock WH, et al 1993; Nadeau KC, et al, 1995). Several are involved in macrophage recruitment; others are characteristic products of activated macrophages but may be synthesized by renal cells under certain circumstances. Macrophages have been implicated as important contributors to graft injury. Inhibition of macrophage function ameliorated graft injury (Azuma H, et al, 1995), whereas interventions designed to increase macrophage activation exacerbated chronic graft injury (Nagano H, et al 1997). The presence of macrophages in CAN has also been associated with the expression of macrophage-derived cytokines, particularly interleukin (IL)1 α , IL-6, TNF- α , MCP-1, and growth factors, including TGF- β and PDGF (Nadeau KC et al. 1995, Azuma H et al. 1997, Heemann UW. et al. 1994). Adhesion molecules are important in the events associated with this process; organs that maintain high levels of intercellular adhesion molecules (ICAM-1), for instance, inevitably progress to CAN (Matas AJ 1994). IL-6, TNF-a, and TGF- may participate in fibrosis by production of mesangial matrix and extracellular matrix, and production of interstitial fibrosis by acting on fibroblasts (Hirano T et al. 1990, Bruijn JA et al. 1994). The best way to prevent CAN may be to regulate the allograft´s local production of cytokines, growth factors, and eicosanoids.

1.6 Age and renal transplantation

The increasing number of patients awaiting kidney transplantation in association with the worldwide donor shortage has led to a more extensive utilization of suboptimal donors, specifically very young and very old ones. Moreover, the relative proportion of elderly among kidney recipients has sharply increased. Therefore, the influence of age on renal

transplantation, both, donor and recipient has gained more and more attention.

Age is considered as an alloantigen-independent risk factor in kidney transplantation (Brenner BM et al. 1993; Waiser J et al. 1997). Donor age may affect allograft function at least in two ways. On the one hand, nephron dose decreases with advancing age (Kaplan C et al. 1975; Kappel B et al. 1980; Gjertson DW 1996); on the other hand, a pediatric kidney is unaccustomed to the workload demanded by an adult recipient (Harmon WE et al. 1992; Yuge J et al.1990; Opelz G 1988; Hayes JM et al. 1991). In these situations, it has been proposed that the reduced functional transplanted renal mass may lead to a hyperfiltration-induced glomerular injury and an inferior long-term outcome. Furthermore, recipient age may be of importance. Young age is believed to be associated with higher (Scornik JC et al. 1996; Ettenger RB 1992), advanced age with attenuated immune responsiveness (Takemoto S et al. 1988).

1.7 Hypothesis

It is difficult to evaluate the influence of donor and recipient age in clinical settings as a large number of other factors influences allograft outcome. Clinical surveys differ in the number of patients studied, inclusion criteria as well as the length of follow-up. In addition, several centers tend to transplant older kidneys into older recipients according to an age-matching policy. Therefore, donor- and recipient-related differences cannot be investigated separately. The inferior results that were observed with very young donors may be due to surgical problems during transplantation (Benedetti E et al 1994). Thus, it is not surprising that some studies conclude that increased donor age is associated with a reduced allograft survival (Takemoto S et al. 1988, Yuge J et al. 1991) while others noted no differences (Sumrani N et

al. 1991; Fauchald P et al. 1991; Zhou YC et al. 1989). Whether or not recipient age has an impact on long-term outcome is even more controversial (Hestin D et al. 1994; Bunchman TE et al. 1994; Gourlay W et al. 1995).

Our present study was designed to determine the effects of donor and recipient age on the development of chronic renal allograft rejection in the absence of other risk factors. Therefore, we have transplanted kidneys from young, adult and old donors into young, adult and old recipients and evaluated functional and morphological changes typically associated with chronically rejecting renal allografts.

2 Materials and Methods

2.1 Animals

Inbred male rats (Charles River, Germany) were used throughout the experiments. Lewis rats (LEW, RT1) acted as graft recipients and Fisher (F344, RTlvl) rats as donors. Animals were kept under standard conditions and were fed rat chow and water ad libitum. All experiments were approved by the local Animal Care and Research Committee.

2.2 Kidney transplantation

The left donor kidney was isolated, cooled and positioned orthotopically into host whose left renal vessels had been dissected free, clamped and the native kidney removed. Donor and recipient artery, vein, and ureter were then anastomosed end-to-end with 10-0 prolene sutures. No ureteral stent was used. The ischemic time ranged from 25 to 30 minutes. All animals were treated with low dose Cyclosporin A (1,5mg/kg/day × 10 days) to suppress an initial episode of acute rejection. The contra lateral native kidney was removed on the 10th postoperative day. Rats with any overt sign of unsuccessful operation were discarded from the experiment.

2.3 Experimental groups

Animals were defined as young (Y), adult (A), and old (O) based on their age at transplantation, which were 8 (Y), 16 (A), and 40 (O) weeks respectively. Animals were assigned to the following 9 experimental groups (n=7/group) according to the age of the donor and recipient: young donor,

young recipient (Y→Y); young donor, adult recipient (Y→A); young donor, old recipient (Y→O); adult donor, young recipient (A→Y); adult donor, adult recipient (A→A); adult donor, old recipient (A→O); old donor, young recipient (O→Y); old donor, adult recipient (O→A); old donor, old recipient (O→O).

2.4 Functional Measurements

Every four weeks, body weight was measured and 24-hour urine samples were collected using metabolic cages with a urine-cooling system. Quantitative urine protein was determined nephelometrically (Boehringer Mannheim, Germany). Serum and urine creatinine levels were measured and creatinine clearance was calculated at the end of the study.

2.5 Harvesting

After 24 weeks rats were anaesthetized with diethylether and intraaortic blood pressure was measured (Sirecust 404; Siemens, Germany). Thereafter, animals were bled and the transplanted kidney was removed. Hematocrit was determined using capillary microcentrifugation. Representative portions of the kidneys were snap-frozen in liquid nitrogen and stored at –80°C for immunohistological and PCR analysis, or fixed in buffered formalin (4%) for histological evaluation.

2.6 Histology

For histology, kidney tissues were fixed in 4 % buffered formalin, embedded in paraffin, and stained with hematoxylin/eosin to evaluate inflammatory infiltrates as well as tubulointerstitial

fibrosis and tubular atrophy. Periodic acid-Schiff (PAS) reaction was performed to evaluate the extent of glomerulosclerosis and graft vasculopathy. Glomerulosclerosis was defined as a collapse of capillaries, adhesion of the obsolescent segment of Bowman's capsule and the entrapment of hyaline in the mesangium (Rennke HG et al. 1989). At least 200 glomeruli were counted per kidney section, and the proportion of sclerosed to total glomeruli was expressed as a percentage. Glomerulopathy, tubular atrophy, interstitial fibrosis and vascular intimal proliferation were quantified according to the Banff´97 classification (Chomczynski P et al. 1987) and scored from 0 to 3, to produce numerical coding (0 – 12 +) of kidney damage.

2.7 Antibodies and immunohistology

Monoclonal antibodies against macrophages (ED1), CD5+ T-lymphocytes (OX19), were purchased from Serotec Camon Labor-Service GmbH (Wiesbaden, Germany). The secondary rabbit anti-mouse antibody and the alkaline phosphatase anti-alkaline phosphatase (APAAP) complex were obtained from Dako A/S (Hamburg, Germany).

Representative portions of kidney grafts were snap-frozen in liquid nitrogen, cut with a cryostat (4μm), fixed in acetone at 4°C for 5 min, air dried, and stained with the respective antibodies. After incubation with the primary antibody, the sections were incubated with rabbit anti-mouse IgG and with the APAAP complex. Positive cell counts for macrophages (ED1) and T-lymphocytes (OX19) were expressed as a mean ± SEM of cells/field of view (c/FV); >20 FV/section/specimen were evaluated at 400x magnification.

2.8 Reverse transcriptase-polymerase chain reaction

Total RNA isolation:

Total RNA was extracted and used for reverse transcriptase-polymerase chain reaction (RT-PCR). Kidney tissue was stored in 500 µl of cold lysis solution containing 4-M guanidine isothiocyanate (Sigma), 25 mM sodium citrate (pH 7.0), 0.1 M β-mercaptoethanol, 0.5% sarcosyl and frozen in liquid nitrogen. Total RNA was extracted from the kidneys according to the modified guanidine-isothiocyanate preparation (26). Briefly, frozen tissues were mixed with 4 ml GITC buffer (4mol/l guanidine isothiocyanate, Sigma) and acid phenol-chloroform (pH 4, Roth), and homogenized. The samples were centrifuged at 1500 g for 10 min at 20°C. The supernatant was added to an equal volume of isopropanol followed by centrifugation. The RNA was purified with the Rneasy, Total RNA Isolations Kit (Qiagen GmbH, Hilden, Germany), and stored at -80°C until further processing. The RNA concentration was measured spectrophotometrically.

Reverse transcription:

RNA was amplified by reverse transcription (RT) with an Oligo $(dT)_{12-18}$ primer (Gibco/BRL, Karlsruhe, Germany). 1 µg of total RNA was added to 0.5 µg of primer. A reaction mixture containing buffer solution (TRIS hydrochloride buffer in a concentration 50 mM/l [pH 8.3], potassium chloride in a concentration of 75 mM/l, magnesium dichloride in a concentration of 5 mM/l, dithiotreitol in a concentration of 5 mM/l; Gibco/BRL, Karlsruhe, Germany), adenosine triphosphate, thymidine triphosphate, guanosine triphosphate, and cytosine triphosphate each in a concentration of 0.2 mM/l (deoxynucleoside triphosphates from Boehringer Mannheim GmbH, Mannheim, Germany), 0.5 µl of 40U/µl of recombinant ribonuclease inhibitor (Promega), and 0.5µl of 200U/µl M-MLV reverse transcriptase (Gibco/BRL, Karlsruhe, Germany) was added and the first chain reaction allowed to proceed (36°C, 1 h). The reaction was halted by heating to 95°C for 5 minutes followed by cooling on ice.

Amplification of specific complementary DNA (cDNA):

Specific cDNA products corresponding to mRNA for TGF-β_1 (Ando T et al. 1995), PDGF-A

chain (Feng L et al. 1993), PDGF-B (Lemstrom KB et al. 1994), ß-actin (Siegling A et al. 1994) were amplified using the polymerase chain reaction. 1 µl from RT reaction was taken for PCR, which was performed in PCR buffer (750mM/l TRIS hydrochloride, pH 9.0, 200 mM/l $(NH_4)_2SO_4$, 0.1% (w/v) Tween 20, 20mM/l magnesium dichloride, (Dianova, Hamburg, Germany) using 0.2mM/l of each deoxynucleoside triphosphates, 1 µM/l of both primers (Eurogentec, Köln, Germany), and 2.5 U thermus Aquaticus (Taq) DNA polymerase (Dianova, Hamburg, Germany). A Perkin-Elmer Thermal Cycler (Model 9600, Perkin-Elmer, Norwalk, CT, USA) was used for amplification with the following sequence profile: initial denaturation at 94°C for 3 minutes followed by 30-35 cycles of three temperature PCR (denaturing, 94°C for 30 seconds; annealing, 55°C for 30 seconds; and extension, 72°C for 30 seconds) ending with a final extension at 72°C for seven minutes and cooling to 4°C.

Gel electrophoresis:

The amplified PCR product was identified by electrophoresis of 10µL sample aliquots on 1.5% agarose gel stained with 0.5µg/ml of ethidium bromide. The sample products were visualized by UV transillumination and the gel was photographed. Specific products were identified by size in relation to a known 1 Kb oligonucleotide DNA ladder (Gibco/BRL, Karlsruhe, Germany) run with each gel. Cytokine cDNA was semiquantitated by densitometric comparison with ß-actin (internal control) from the same sample after the positive image was digitized by video for computerized densitometry. The results are given as the ratio of intensity of growth factors to ß-actin mRNA ± SEM.

2.9 Statistical analysis

The effects of donor and recipient age were analysed by MANOVA and linear correlation analysis. Parametric data were compared using ANOVA as appropriate. Non-parametric

distribution values were analysed by Mann-Whitney test. Statistical difference was accepted at $p<0.05$.

3 Results

3.1 Animals

At the beginning of the study, body weights of recipients considerably differed between the groups. However, by 24 weeks, body weights had increased to the same level in all groups. As expected, donor kidney weight highly correlated with donor body weight at the time of transplantation. By contrast, kidney weight did not differ among the groups at the time of harvesting (Fig 1-2) (Tab 1).

At the time of transplantation, donor kidney weight/recipient body weight (KW/BW) ratio in young recipients was considerably higher as compared to adult and old recipients irrespective of whether young recipients received a graft from young, adult or old donors. Moreover, there was a trend towards higher KW/BW ratios in groups A→A (adult recipients with an adult kidney graft), A→O (old recipients with an adult kidney graft), O→A (adult recipients with an old kidney graft) and O→O (old recipients with an old kidney graft) as compared to groups Y→A (adult recipients with an young kidney graft) and Y→O (old recipients with a young kidney graft). Again, at the end of the follow-up period, KW/BW ratio did not differ between the groups (Fig 3) (Tab 1).

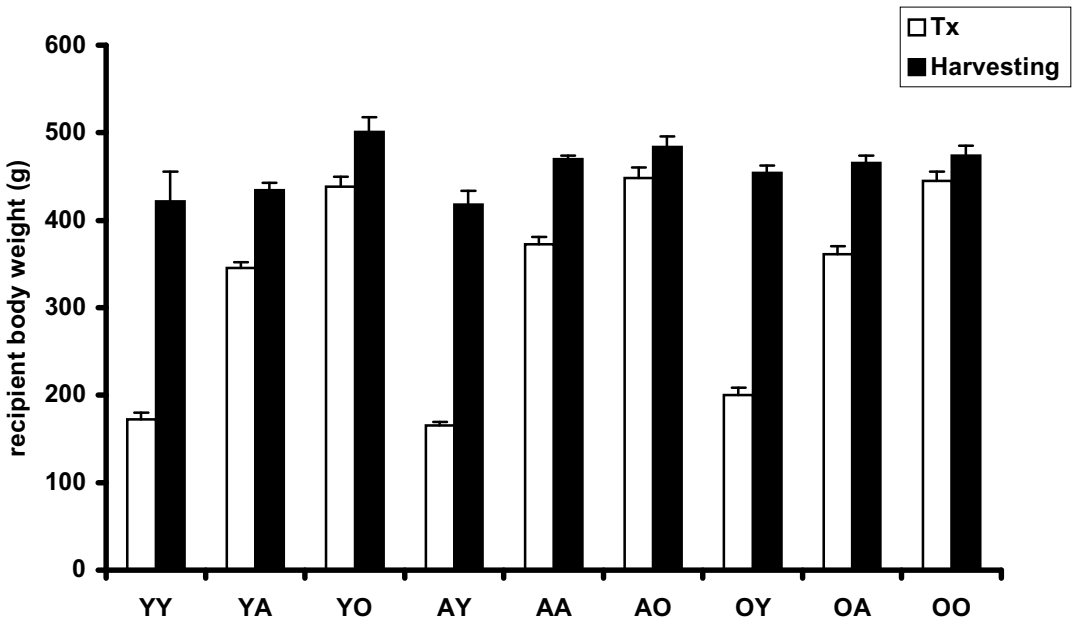


Figure 1

Changes in recipient body weight at the time of transplantation (blank column) and by harvesting (black column). Data are mean±SEM.

Abbreviations: YY: young donor to young recipient; YA: young donor to adult recipient; YO: young donor to old recipient; AY: adult donor to young recipient; AA: adult donor to adult recipient; AO: adult donor to old recipient; OY: old donor to young recipient; OA: old donor to adult recipient; OO: old donor to old recipient. Tx: transplantation.

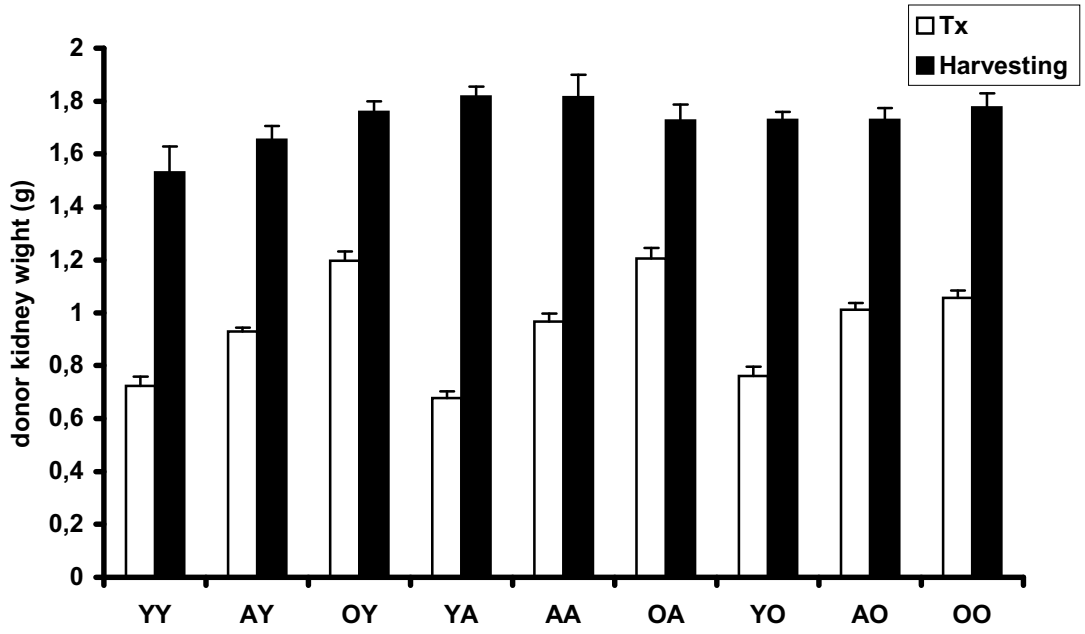


Figure 2

Changes in donor kidney weight at the time of transplantation (donor right kidney, blank column) and by harvesting (graft, black column). Data are mean±SEM.

Abbreviations: YY: young donor to young recipient; YA: young donor to adult recipient; YO: young donor to old recipient; AY: adult donor to young recipient; AA: adult donor to adult recipient; AO: adult donor to old recipient; OY: old donor to young recipient; OA: old donor to adult recipient; OO: old donor to old recipient. Tx: transplantation.

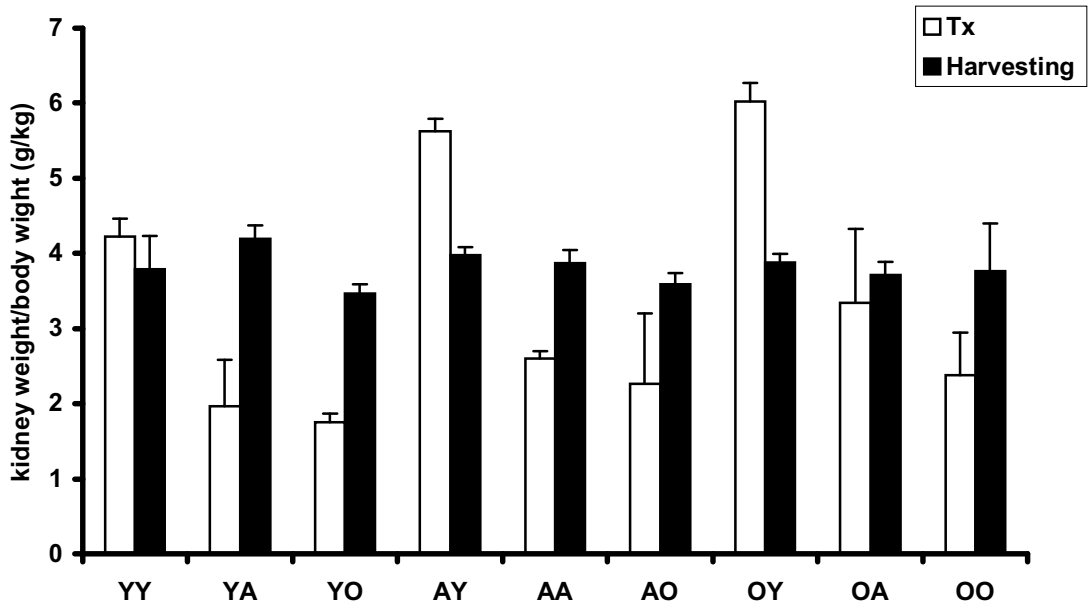


Figure 3

Changes in kidney weight/body weight ratio at the time of transplantation (donor right kidney weight/recipient body weight, blank column) and by harvesting (graft weight/recipient body weight, black column). Data are mean±SEM.

Abbreviations: YY: young donor to young recipient; YA: young donor to adult recipient; YO: young donor to old recipient; AY: adult donor to young recipient; AA: adult donor to adult recipient; AO: adult donor to old recipient; OY: old donor to young recipient; OA: old donor to adult recipient; OO: old donor to old recipient. Tx: transplantation.

Table 1. Kidney weights and body weights

Group	N	Donor body weight (g)	Donor right kidney weight (mg)	Recipient body weight at Tx (g)	Kidney weight / body weight at Tx	Graft weight at harvesting (mg)	Recipient body weight at harvesting (g)	Kidney weight/ body weight at harvesting
Y→Y	6	175 ± 9	722 ± 35	172 ± 8	4.22 ± 0.24	1530 ± 98	422 ± 34	3.79 ± 0.44
Y→A	6	168 ± 3	677 ± 16	345 ± 7	1.96 ± 0.62	1817 ± 51	435 ± 8	4.19 ± 0.18
Y→O	7	198 ± 6	761 ± 36	438 ± 1	1.15 ± 0.12	1730 ± 41	501 ± 15	3.46 ± 0,12
A→Y	7	281 ± 7	929 ± 25	165 ± 4	5.62 ± 0.17	1654 ± 37	418 ± 16	3.98 ± 0.10
A→A	6	289 ± 4	967 ± 30	372 ± 8	2.60 ± 0.10	1815 ± 84	470 ± 4	3.87 ± 0.18
A→O	7	296 ± 5	1011± 41	448 ± 12	2.26 ± 0.94	1730 ± 59	483 ± 12	3.59 ± 0.15
O→Y	7	348 ± 6	1197± 35	200 ± 8	6.01 ± 0.25	1759 ± 28	454 ± 8	3.88 ± 0.11
O→A	7	349 ± 8	1204± 24	361 ± 9	3.34 ± 0.98	1727 ± 43	465 ± 8	3.71 ± 0.91
O→O	7	336 ± 8	1057± 26	445 ± 10	2.38 ± 0.57	1776 ± 53	473 ± 10	3.77 ± 0.17

Donor body weight, donor right kidney weight, recipient body weight at Tx and kidney weight/ body weight ratio at Tx, were determined at the time of transplantation; graft weight, recipient body weight, and kidney weight/body weight at harvesting were determined at 24 weeks after transplantation. Abbreviations: Y→Y: young donor to young recipient; Y→A: young donor to adult recipient; Y→O: young donor to old recipient; A→Y: adult donor to young recipient; A→A: adult donor to adult recipient; A→O: adult donor to old recipient; O→Y; old donor to young recipient; O→A: old donor to adult recipient; O→O: old donor to old recipient; Tx: transplantation. Results are given as mean ± SEM.

3.2 Functional parameters

Donor and recipient age did not significantly affect mean arterial blood pressure and hematocrit. However, a correlation was observed between recipient body weight at transplantation and creatinine clearance ($r=0.284$; $p=0.028$). No significant correlation was found between creatinine clearance and donor kidney weight, or KW/BW ratio at the time of transplantation. There was a significant increase in proteinuria and decrease in creatinine clearance in Y→O (old recipients of a young kidney graft) as compared to Y→Y (young recipients of a young kidney graft) (Fig 4, 5). There was a trend towards increased proteinuria and decreased creatinine clearance in Y→O as well as recipient groups of the same age (A→O, O→O), but the differences did not reach statistical significance. Interestingly, the group Y→Y developed the lowest proteinuria and the highest creatinine clearance as compared to the other groups (Tab 2).

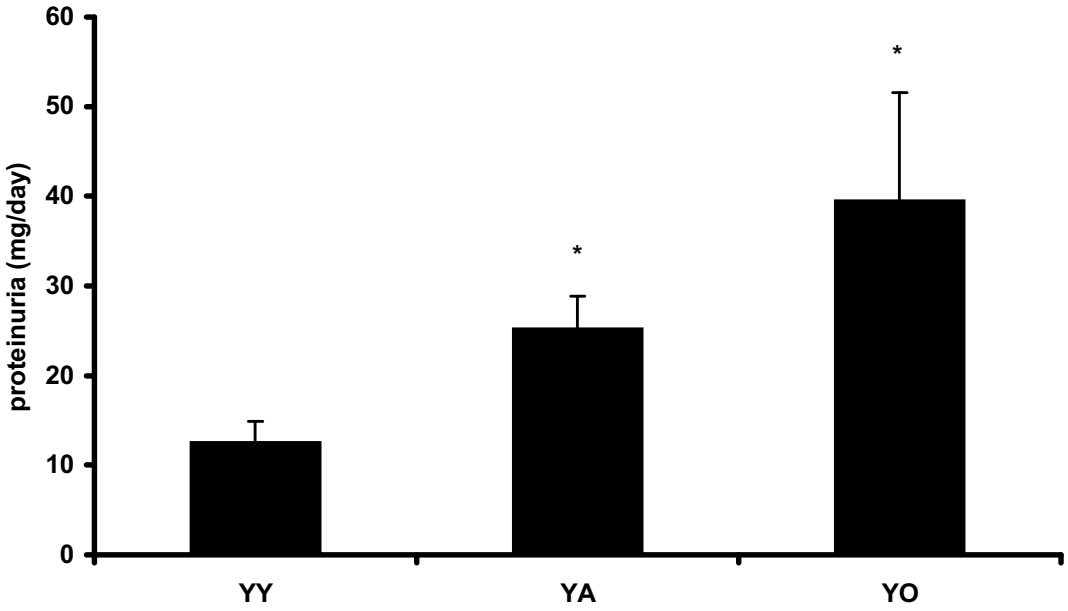


Figure 4

24-hour urinary protein excretion 24 weeks after kidney transplantation in young, adult, old recipients of graft from young donor. Data are given as mean ± SEM. *: $p<0.05$ vs. young recipients with a kidney graft from young donor (YY).

Abbreviations: YY: young donor to young recipient; YA: young donor to adult recipient; YO: young donor to old recipient.

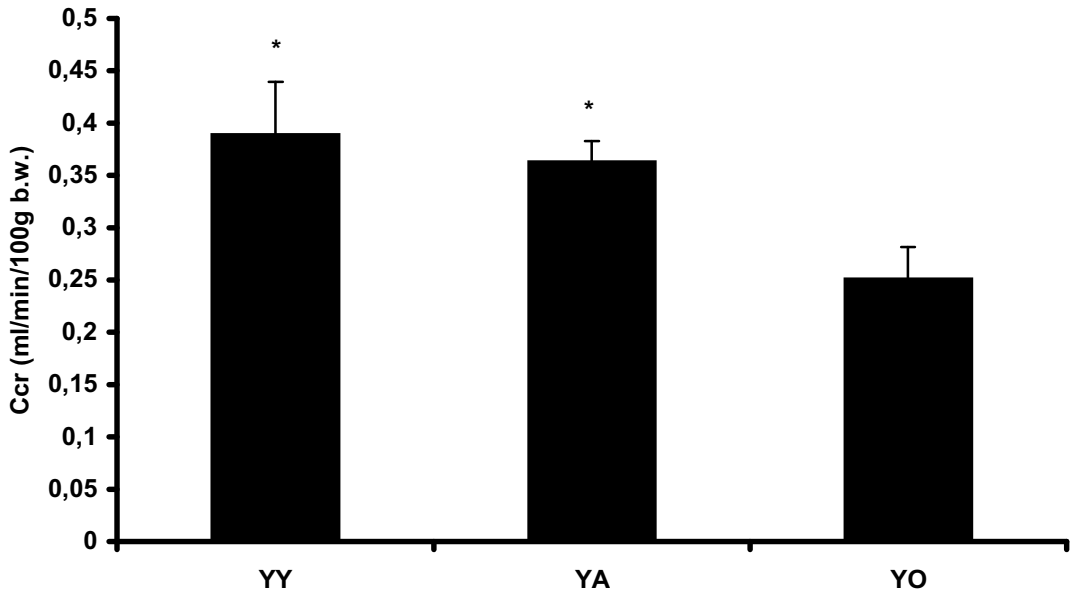


Figure 5

Creatinine clearance 24 weeks after kidney transplantation in young, adult, old recipients of graft from young donor. Data are given as mean ± SEM. *: $p<0.05$ vs. old recipients with a kidney graft from young donor (YO).

Abbreviations: YY: young donor to young recipient; YA: young donor to adult recipient; YO: young donor to old recipient.

Table 2. Functional parameters at harvesting (24 weeks after transplantation)

Group	N	Proteinuria (mg/day)	Creatinine clearance (ml/min/100g b.w.)	Mean arterial blood pressure (mmHg)	Hematocrit %
Y→Y	6	12.5 ± 2.4	0.39 ± 0.05	85.5 ± 7.2	42.6 ± 0.7
Y→A	6	25.2 ± 3.6	0.36 ± 0.02	71.8 ± 5.5	41.6 ± 1.2
Y→O	7	39.5 ± 12.1	0.25 ± 0.03	84.7 ± 9.4	37.9 ± 1.9
A→Y	7	28.5 ± 2.7	0.35 ± 0.01	91.6 ± 3.6	43.2 ± 1.4
A→A	6	34.7 ± 4.0	0.33 ± 0.01	75.2 ± 8.3	36.1 ± 3.8
A→O	7	24.1 ± 5.9	0.33 ± 0.01	90.1 ± 11.9	39.7 ± 0.8
O→Y	7	30.8 ± 6.1	0.36 ± 0.01	80.9 ± 10.4	39.2 ± 1.2
O→A	7	27.8 ± 2.9	0.31 ± 0.02	84.0 ± 10.4	42.5 ± 0.7
O→O	7	27.4 ± 7.2	0.37 ± 0.04	94.0 ± 14.4	38.5 ± 1.9

Abbreviations: Y→Y: young donor to young recipient; Y→A: young donor to adult recipient; Y→O: young donor to old recipient; A→Y: adult donor to young recipient; A→A: adult donor to adult recipient; A→O: adult donor to old recipient; O→Y; old donor to young recipient; O→A: old donor to adult recipient; O→O: old donor to old recipient; b.w.: body weight. Results are given as mean ± SEM.

3.3 Histology

At the end of the follow-up, histological evaluation revealed glomerulosclerosis, tubular atrophy, interstitial fibrosis, inflammatory cellular infiltration and intimal thickening of graft arteries in all groups. The severity of glomerulosclerosis tended to increase with recipient age disregarding donor age. In animals with a kidney allograft derived from an old donor, recipient age determined allograft outcome. Particularly the glomerulosclerosis index was significantly higher in old and adult recipients (O→O, O→A) than in young recipients (O→Y) (Fig 6). The profound glomerular injury in these animals was accompanied by a significantly higher degree of interstitial fibrosis and a moderate tubular atrophy, observed in approximately 25% of the cortical tubuli. In addition, the luminal obliteration of graft arteries (approximately 25%) was more obvious in these allografts. We noted similar effects of the recipient age on the overall picture of chronic rejection in animals that had received a graft of an adult donor, with mild interstitial fibrosis, mild tubular atrophy and mild to moderate intimal thickening. However, these differences did not reach statistical significance. Accordingly, in animals that received a kidney of a young donor, the percentage of sclerosed glomeruli was significantly higher in old recipients (Y→O) as compared to young recipients (Y→Y) (Fig 7).

No significant differences were noted either in interstitial fibrosis, tubular atrophy or intimal proliferation between recipients with respect to donor age although old recipients of a graft from an old donor tended to have relatively more glomerulosclerosis than those with grafts from young or adult donors. However, the differences were not significant (Fig 8a-c) (Tab 3).

While the influence of recipient age was mild, recipient body weight and kidney weight/body weight ratio significantly correlated with the percentage of sclerotic glomeruli at the day of transplantation (Tab 4). However, at the time of harvesting, we observed no significant

correlation between recipient body weight or KW/BW ratio and morphological results.

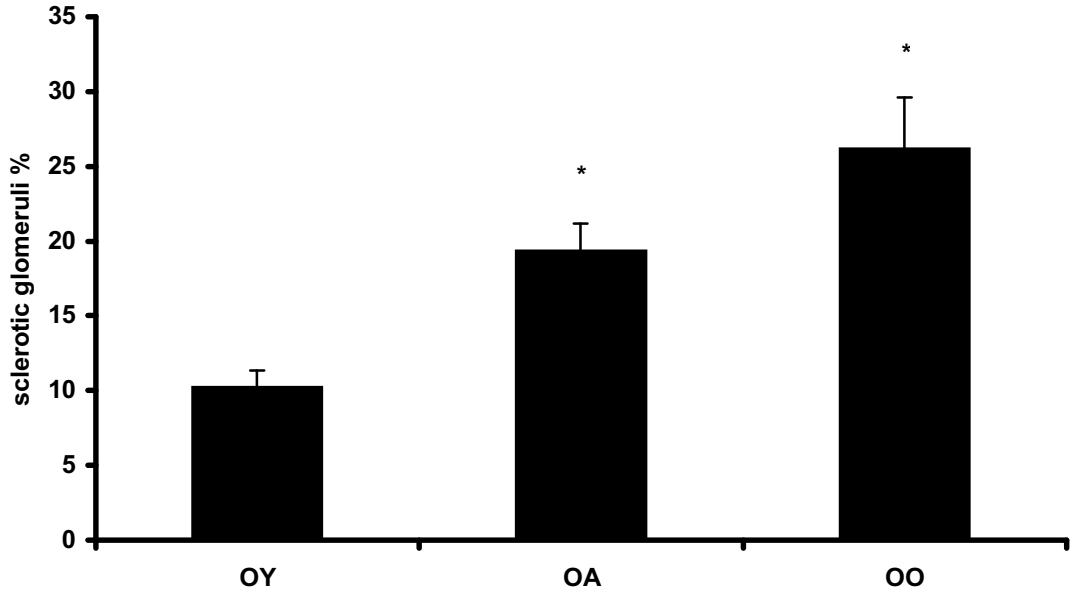


Figure 6

Percentage of sclerotic glomeruli in young, adult, old recipients with a kidney allograft from an old donor. Data are mean ± SEM. *: $p<0.05$ vs. young recipients with a kidney graft from old donor (OY).

Abbreviations: OY: old donor to young recipient; OA: old donor to adult recipient; OO: old donor to old recipient.

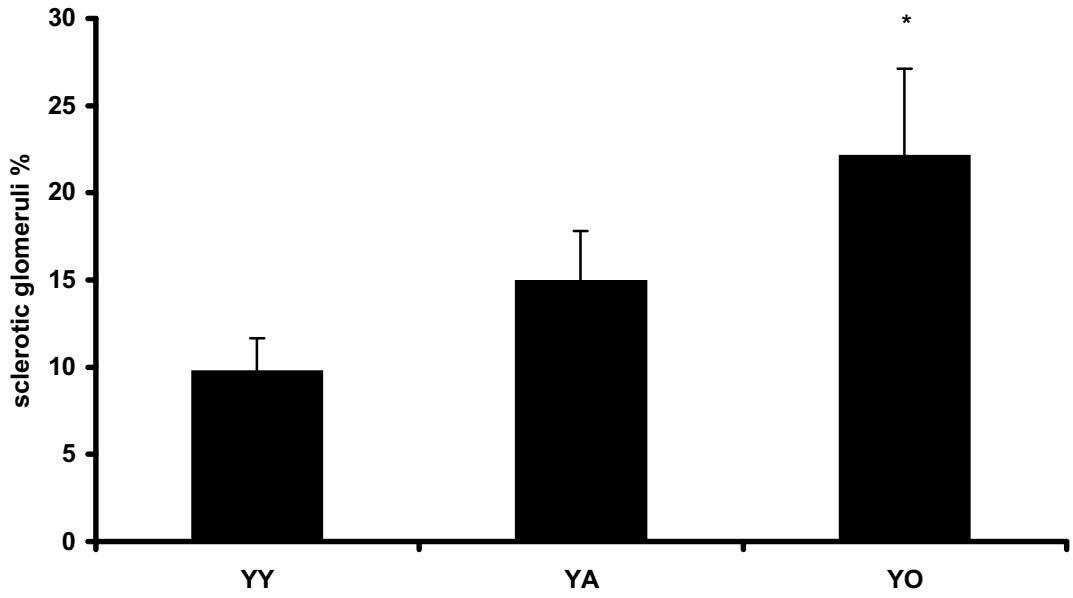
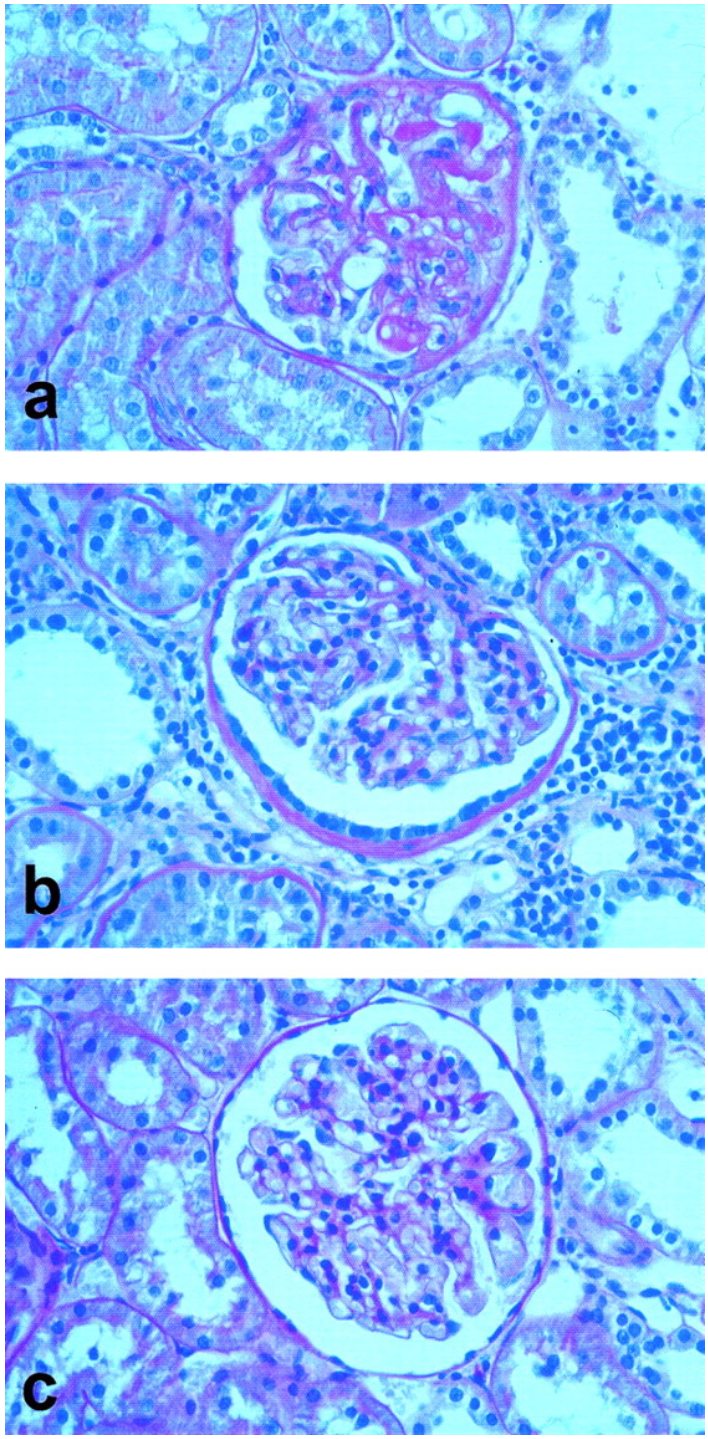


Figure 7

Percentage of sclerotic glomeruli in young, adult, old recipients with a kidney allograft from a young donor. Data are mean ± SEM. *: $p<0.05$ vs. young recipients with a kidney graft from young donor (YY).

Abbreviations: YY: young donor to young recipient; YA: young donor to adult recipient; YO: young donor to old recipient.

Figure 8:



Representative photomicrographs of periodic acid-Schiff-stained renal allograft sections: (a) old donor to old recipient; (b) young donor to old recipient; (c) young donor to young recipient.

Table 3. Immunohistological and histological results in allografts

Group	N	Macrophages (ED1+) (cells/fv)	Lymphocytes (OX19+) (cells/fv)	Sclerotic glomeruli (%)	Banff score 0-12 +
Y→Y	6	7.8 ± 2.1	10.6 ± 1.2	9.8 ± 1.9	2.0 ± 0.6
Y→A	6	18.2 ± 2.1	21.2 ± 2.1 [b]	14.9 ± 2.9	3.0 ± 0.7
Y→O	7	7.1 ± 1.4 [d]	9.9 ± 1.0 [d]	22.1 ± 5.0 [b]	2.3 ± 0.8
A→Y	7	8.1 ± 1.3	10.9 ± 1.8	12.5 ± 2.3	2.4 ± 0.4
A→A	6	16.4 ± 1.6	16.8 ± 3.2	17.3 ± 3.6	3.7 ± 1.2
A→O	7	20.6 ± 5.3 [a b]	19.4 ± 2.4 [a]	18.9 ± 2.3	3.1 ± 0.9
O→Y	7	5.7 ± 0.6	11.0 ± 0.9	10.2 ± 1.1	1.3 ± 0.4 [c]
O→A	7	17.2 ± 2.1 [b]	17.4 ± 2.0	19.4 ± 1.8 [b]	3.4 ± 0.6 [b]
O→O	7	35.8 ± 5.3 [a b c d]	33.6 ± 6.3 [a b c d]	26.2 ± 3.4 [b]	4.0 ± 0.6 [b]

Abbreviations: Y→Y: young donor to young recipient; Y→A: young donor to adult recipient; Y→O: young donor to old recipient; A→Y: adult donor to young recipient; A→A: adult donor to adult recipient; A→O: adult donor to old recipient; O→Y: old donor to young recipient; O→A: old donor to adult recipient; O→O: old donor to old recipient; b.w.: body weight. Results are given as mean ± SEM.

[a] $p < 0.05$ vs. young donor based on recipient of same age level;[b] $p < 0.05$ vs. young recipient based on donor of same age level ;

[c] $p < 0.05$ vs. adult donor based on recipient of same age level;[d] $p < 0.05$ vs. adult recipient based on donor of same age level.

3.4 Immunohistology

Immunohistological analysis of graft tissues revealed mononuclear cell infiltration in all groups, localizing preferentially in perivascular and periglomerular areas. Recipient age significantly influenced the number of infiltrating CD5+ T-lymphocytes and ED1-positive macrophages when kidneys from old donors were transplanted. Infiltration of T-lymphocytes and macrophages was significantly more pronounced in group O→O than in O→A and O→Y (Fig 9, 10). Similar effects of recipient age were noted in animals who had received a kidney from an adult donor. However, differences did not reach statistical significance. If young donors were utilized, cellular infiltration was most pronounced in adult recipients (Y→A). The number of infiltrating cells was low in young recipients with a young kidney graft (Y→Y) and old recipients with a young kidney graft (Y→O) (Tab 3).

Increasing donor age did not influence the number of CD5+ T-lymphocytes and macrophages in young or adult recipients. However, in old recipients cellular infiltration increased with donor age; it was most pronounced in old recipients of an old kidney graft (O→O) (Fig 11, 12) (Tab 3).

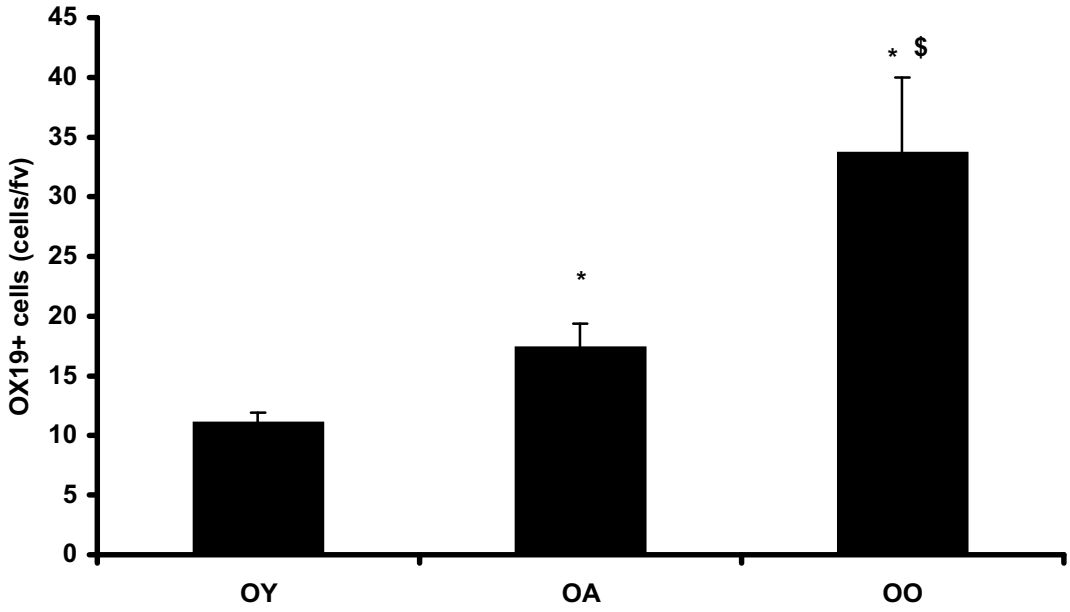


Figure 9

Immunohistochemical evaluation of CD5-positive cellular infiltration in young, adult, old recipients with a kidney allograft from an old donor. Data are mean ± SEM. *: $p<0.05$ vs. young recipients with a kidney graft from old donor (OY); $^{\$}$: $p<0.05$ vs. adult recipients with a kidney graft from old donor (OA).

Abbreviations: OY: old donor to young recipient; OA: old donor to adult recipient; OO: old donor to old recipient.

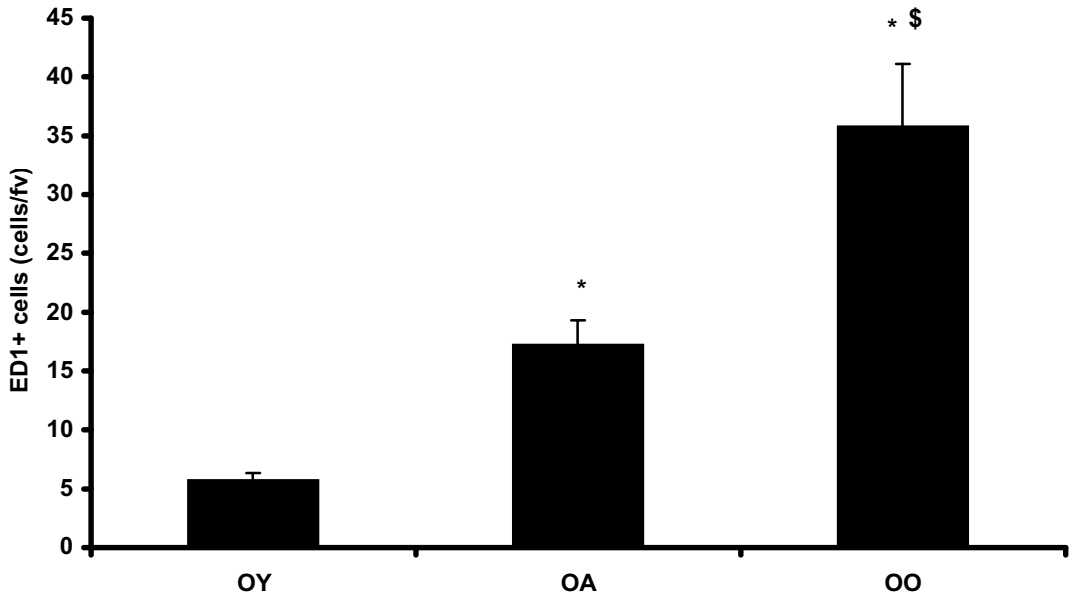


Figure 10

Immunohistochemical evaluation of ED1-positive cellular infiltration in young, adult, old recipients with a kidney allograft from an old donor. Data are mean ± SEM. *: $p<0.05$ vs. young recipients with a kidney graft from old donor (OY); $^{\$}$: $p<0.05$ vs. adult recipients with a kidney graft from old donor (OA).

Abbreviations: OY: old donor to young recipient; OA: old donor to adult recipient; OO: old donor to old recipient.

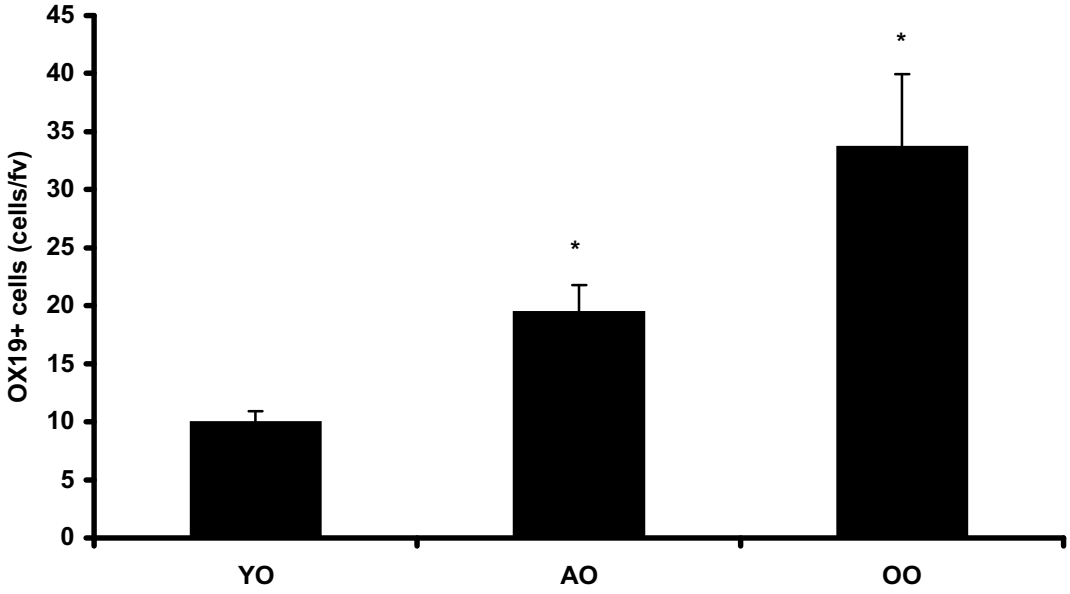


Figure 11

Immunohistochemical evaluation of CD5-positive cellular infiltration in old recipients with a kidney allograft from young, adult, old donor. Data are mean ± SEM. *: $p<0.05$ vs. old recipients with a kidney graft from young donor (YO).

Abbreviations: YY: young donor to young recipient; YA: young donor to adult recipient; YO: young donor to old recipient.

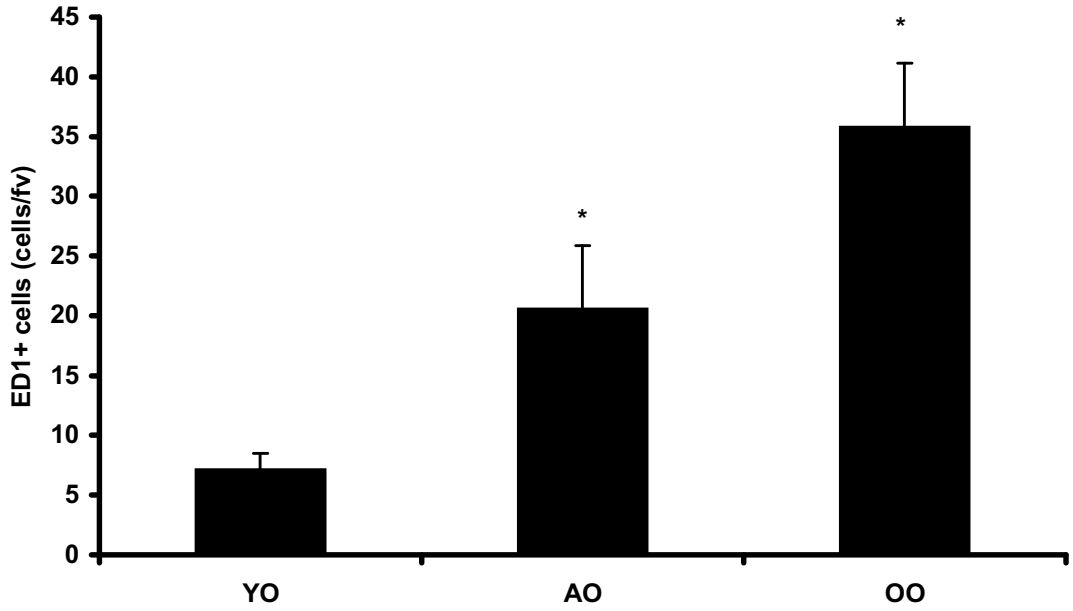


Figure 12

Immunohistochemical evaluation of ED1-positive cellular infiltration in old recipients with a kidney allograft from young, adult, old donor. Data are mean ± SEM. *: $p<0.05$ vs. old recipients with a kidney graft from young donor (YO).

Abbreviations: YY: young donor to young recipient; YA: young donor to adult recipient; YO: young donor to old recipient.

Table 4. Correlation of body and kidney weight at transplantation with functional and morphological parameters

	Proteinuria (mg/day)		Creatinine clearance (ml/min/100 g b.w.)		Sclerotic glomeruli %	
Recipient body weight at Tx	0.176	(0.179)	-0.284*	(0.028)	0.507**	(0.000)
Donor kidney weight at Tx	0.082	(0.533)	0.079	(0.550)	0.145	(0.291)
Kidney weight/body weight at Tx	-0.099	(0.453)	0.242	(0.062)	-0.374**	(0.005)
Recipient body weight at harvesting	0.158	(0.228)	-0.603**	(0.000)	0.223	(0.102)
(graft) kidney weight at harvesting	0.118	(0.371)	0.030	(0.821)	0.221	(0.105)
Kidney weight/body weight at harvesting	-0.096	(0.468)	0.561**	(0.000)	-0.052	(0.705)

All data are given as the value of correlation coefficient and (p). Tx: transplantation.

* Correlation is significant at the 0.05 level.

** Correlation is significant at the 0.01 level.

3.5 Reverse transcriptase-polymerase chain reaction

We observed a trend towards an increased intragraft mRNA expression of PDGF-A, -B chain and TGF-ß in old recipients as compared to young recipients who had received a kidney of the same donor age. A significantly increased expression of these factors correlating to recipient age was only observed among groups receiving an adult graft (Fig 13-15). Donor age per se did not significantly affect allograft growth factor expression.

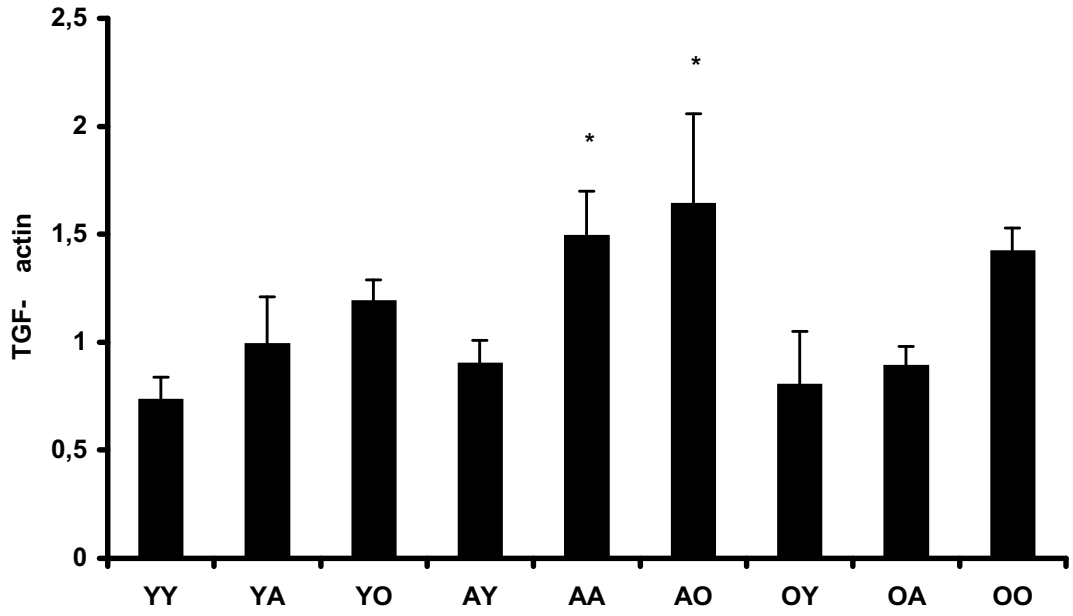


Figure 13

Growth factor TGF-β/β-actin mRNA expression by RT-PCR in renal allografts 24 weeks after transplantation. *: $p<0.05$ vs. AY. All the results are given as mean ± SEM.

Abbreviations are: YY: young donor to young recipient; YA: young donor to adult recipient; YO: young donor to old recipient; AY: adult donor to young recipient; AA: adult donor to adult recipient; AO: adult donor to old recipient; OY: old donor to young recipient; OA: old donor to adult recipient; OO: old donor to old recipient.

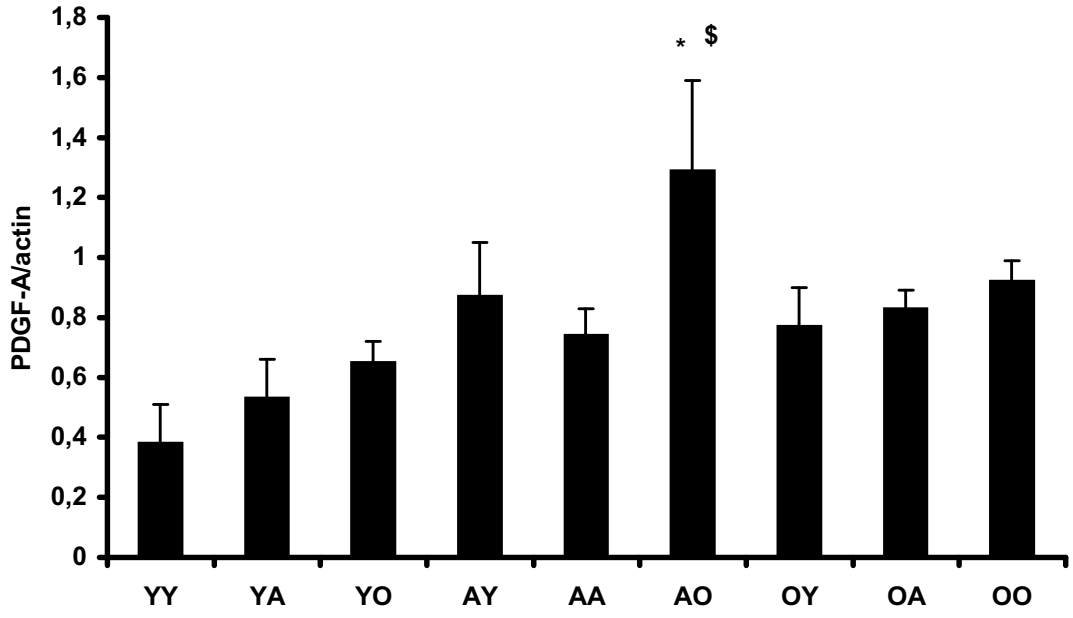


Figure 14

Growth factor PDGF-A /β-actin mRNA expression by RT-PCR in renal allografts 24 weeks after transplantation. *: $p<0.05$ vs. AY; $: $p<0.05$ vs. AA. All the results are given as mean ± SEM. Abbreviations are: YY: young donor to young recipient; YA: young donor to adult recipient; YO: young donor to old recipient; AY: adult donor to young recipient; AA: adult donor to adult recipient; AO: adult donor to old recipient; OY: old donor to young recipient; OA: old donor to adult recipient; OO: old donor to old recipient.

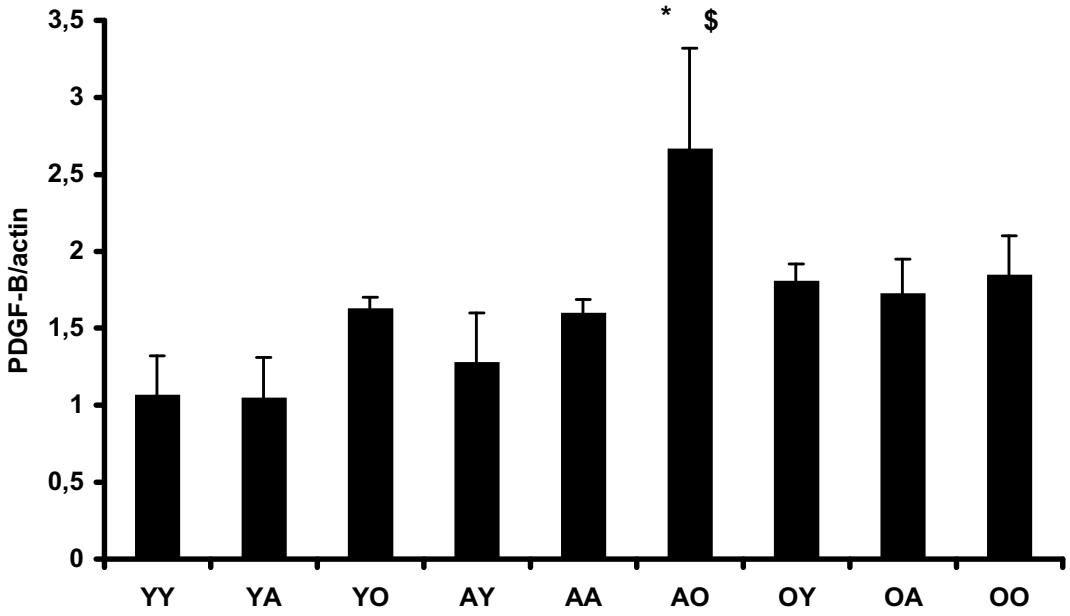


Figure 15

Growth factor PDGF-B /β-actin mRNA expression by RT-PCR in renal allografts 24 weeks after transplantation. *: $p<0.05$ vs. AY; $: $p<0.05$ vs. AA. All the results are given as mean ± SEM. Abbreviations are: YY: young donor to young recipient; YA: young donor to adult recipient; YO: young donor to old recipient; AY: adult donor to young recipient; AA: adult donor to adult recipient; AO: adult donor to old recipient; OY: old donor to young recipient; OA: old donor to adult recipient; OO: old donor to old recipient.

4 Discussion

Donor and recipient age may influence many aspects of kidney transplantation including chronic rejection. It has been postulated for many years that a mismatch between donor kidney supply (kidney weight) and functional demands of the recipient (body weight) result in a hyperfiltration-induced glomerular injury, which may significantly influence long-term outcome after kidney transplantation (Brenner BM et al. 1993; Heemann U et al. 1994; Azuma H et al. 1997). From the recipient point of view, our results are consistent with this notion.

In our experiment, advanced recipient age was associated with more severe allograft injury. The best outcome was achieved in young recipients irrespective of whether allografts were derived from young, adult or old donors. By contrast, kidney grafts in old recipients developed serious renal damage independent of donor age. Moreover, initial recipient body weight correlated with the development of chronic rejection. A significant but less profound correlation was observed between donor kidney weight and recipient body weight ratio at the time of transplantation and the development of allograft injury. Donor kidney weight by itself had no significant correlation with long-term allograft injuries, which may have lead to the less profound influence of KW/BW ratio on the long-term allograft outcome in our experiment. Therefore, factors other than kidney weight have to be taken into consideration, donor and recipient age among them. In other words, the growth in recipient body weight could reflect higher metabolic demand with aging, while increased donor kidney weight may reflect tubular but not nephron mass; the increased kidney weight of aging rats may mask the reduced number of functioning nephrons. Thus, when we take KW/BW ratio as a reference for renal transplantation, aging-related nephron loss or functional deterioration should be taken into consideration.

In our experiment, the impact of donor age on the development of chronic renal rejection was

weak. Donor age influenced allograft outcome only in old recipients, that was, increased donor age was associated with a more rapid progression of chronic rejection. In young and adult recipients, donor age had no effect on allograft outcome. Such findings were unexpected, as lower survival rates have been reported in a number of clinical studies utilizing allografts from old donors (Takemoto S et al. 1988, Yuge J et al. 1991).

Experimental studies in rats as well as in humans have clearly suggested that age is accompanied by several morphological changes in the kidney. In rats, the earliest indications of age related kidney damage occur at three months. Typical lesions include thickening of the glomerular basement membrane (Terasaki PI et al.1990; Hirokawa K 1975), mesangial proliferation (Bell RH Jr et al. 1984), the fusion of foot processes of podocytes (Halloran PF et al. 1999), and glomerular sclerosis (Bell RH Jr et al. 1984; Gray JE 1977; Couser WG et al. 1975). Moreover, it has been suggested that old kidneys are more susceptible to ischemia/reperfusion injury and renal damage (Halloran PF et al. 1999). These injuries would in turn contribute to a cascade of inflammatory events and further reduce nephron mass. In the light of our findings, these age-related morphological changes in the kidney may not trigger the rejection process. However, in old recipients of old donor kidneys there was a trend towards a more pronounced allograft injury. Therefore, it is reasonable to assume that small differences between the donors become more obvious in animals with a high metabolic demand, that is, in old recipients.

It has been well established that protein restriction ameliorates, while high dietary protein intake exacerbates glomerular injury (Anderson S et al. 1986). As the rats in our experiment were not fed according to a protein restriction diet, it is likely that their growth was accompanied by an increased protein intake. The “high protein diet” in older recipients may have contributed to the more severe allograft injury in these groups. Similarly, in young recipients low protein intake may be responsible for the better outcome of these grafts.

In humans and rodents, immune functions decline with age (Hirokawa K 1985; Hirokawa K 1988;

Makinodan T et al.1980). The involution of the thymus may be responsible (Hirokawa K 1988). In our experiment, we did not observe any beneficial effects of lower alloresponsiveness in old recipient. The best outcome was observed in young recipients of a kidney of a young donor. Clinically, poor outcome is observed in young recipients (Arbus GS et al. 1991). There are a couple of possibilities to explain this difference. First of all, young recipients tend to have a lower compliance than older ones in a clinical setting. Furthermore, we did not look for acute effects but for chronic ones. In this setting it may be of importance that age related changes in subsets of CD4 T cells in the periphery shift from TH1 to TH2. While the TH2 cells are helpful for acute rejection they may be of importance for the initiation and maintenance of chronic allograft nephropathy (Shirwan H 1999, Hirokawa K 1992).

PDGF and TGF-β are likely to be involved in the development of chronic allograft nephropathy (Hutchinson IV 1999; Haller H et al. 1997). TGF-β has the potential to induce pathological changes such as fibrosis and arteriosclerosis (Hutchinson IV 1999). The effects of PDGF include promotion of hypertrophy and proliferation, stimulation of chemotaxis and contraction in vascular beds, and stimulation of TGF-β production (Abboud HE 1992). In rats, it has been demonstrated that decreased renal mass accelerates and intensifies changes resembling chronic rejection, in parallel to an increased expression of PDGF and TGF-β, adhesion molecules, and endothelin (Haller H et al. 1997, Kingma I et al.1993). In our experiment, higher expression of PDGF-A and B chain as well as TGF-β in old recipients of an adult or old graft paralleled immunological and histological results. Therefore, the increased expression of these growth factors may to some extent mediate age-related allograft injury.

In conclusion, the influence of recipient age and body weight on allograft injury was more pronounced than that of donor kidney weight in our experiment.

5 Conclusion

In summary, our results demonstrate that recipient age and body weight profoundly influence the long-term renal allograft outcome in our rat model. Increased recipient age and body weights are detrimental to the graft, implicating the importance of recipient functional demand in the development of chronic allograft nephropathy. By contrast, donor kidney weight did not significantly affect the late allograft injury. Only in old recipients groups, increase in donor age is associated with the progression of chronic allograft nephropathy, suggesting that increased recipient functional demand may also augment the influence of donor- aging-related nephron loss in late allograft failure.

6 Summary

Nephron dose and immune response change with age. Thus, age is a potential risk factor for graft survival after kidney transplantation. The aim of the current study was to determine whether age-related differences are of importance for the long-term outcome after renal transplantation. Kidneys of Fisher (F344) rats were orthotopically transplanted into nephrectomized Lewis (LEW) rats. Kidneys were transplanted in donors and recipients of three age levels: young (Y: 8 weeks old), adult (A: 16 weeks old), and old (O: 40 weeks old). Rats were harvested 24 weeks after transplantation and functional, morphological, and molecular evaluations were performed. Recipient rather than donor age determined graft survival. No significant correlation was found between donor kidney weight at the day of transplantation and morphological results. Advanced recipient age was associated with reduced creatinine clearance, more severe histological injuries including extended glomerular sclerosis, interstitial fibrosis, vascular lesions, more pronounced cellular infiltration, as well as a higher expression of TGF-β, and PDGF A and B chain. However, while we observed no significant correlation between donor age or kidney weight at the day of transplantation and morphological results, there was a significant correlation between recipient body weight at the day of transplantation and allograft injury. Therefore, recipient age and weight affect chronic allograft nephropathy. Renal allografts may benefit from young recipient age but may deteriorate in old recipients, suggesting effects of recipient functional demand on long-term outcome.

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8 Abbreviation

APAAP:	alkaline phosphatase anti-alkaline phosphatase
BW:	body weight
CAN:	chronic allograft nephropathy
CsA:	cyclosporin A
DNA:	deoxyribonucleic acid
GAPDH:	glycerinaldehyde-3-phosphate-dehydrogenase
GITC:	guanidine isothiocyanate
ICAM:	intracellular adhesion molecule
IL:	interleukin
INF:	interferon
KW:	kidney weight
MABP:	mean arterial blood pressure
MCP:	monocyte chemotactic protein
MHC:	major histocompatibility complex
PAS:	periodic acid-schiff
PCR:	polymerase chain reaction
PDGF:	platelet-derived growth factor
P_{GC}:	glomerular capillary hydraulic pressure
PP^{125FAK}:	a focal adhesion kinase gene encoded protein tyrosine kinase
RNA:	ribonucleic acid
RT:	reverse transcription
SMCs:	smooth muscle cells
TNF-α:	tumor necrosis factor-α

TGF-ß:	transforming growth factor-ß
UNOS:	United Network For Organ Sharing
UTP:	uriddine-triphosphate
VCAM:	vascular cell adhesion molecule

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